Age Estimation of Mexican Fruit Fly (Diptera: Tephritidae) Based on Accumulation of Pterins

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ABSTRACT A common method of aging adult flies, fluorescence spectrometry, was used to monitor the increase of overall pterine titer in head extracts of *Anastrepha ludens* (Loew). Accumulation of fluorescent compounds was measured as a function of chronological age of flies maintained at 17 and 27°C. Although relative fluorescence increased with age, field studies revealed that this phenomenon could not be used for accurate age estimation, as relative fluorescence did not increase predictably with age over the entire life span. Accumulation of individual pterins, deoxysepiapterin and sepiapterin, were studied in a similar manner. These two specific compounds were separated by high-pressure liquid chromatography and their accumulation was followed at 15 and 30°C in the laboratory and under caged field conditions. While titer of deoxysepiapterin increased steadily in a curvilinear fashion, sepiapterin quickly reached a maximum and then maintained a constant level for the rest of the life of the flies. Based on the physiological response of deoxysepiapterin to chronological time and ambient thermal conditions, this compound was determined to be an age specific biological parameter for the Mexican fruit fly and should allow age estimation in field-collected flies.

KEY WORDS *Anastrepha ludens*, fluorescence, sepiapterin, deoxysepiapterin, age

INFORMATION ON THE age distribution of insects is essential for understanding the population dynamics, biology, and behavior of many species. To assess population age structure in adult insects, several methods have been developed (reviewed by Tyndale-Biscoe 1984); however, none of these methods have been fully effective for assessing the age of adult Mexican fruit flies, *Anastrepha ludens* (Loew).

Anastrepha ludens is a pest of various commercial crop plants, primarily fruits and vegetables. This pest is often spread accidentally to new areas by the shipment of infested agricultural commodities. Insecticide sprays or baits and the sterile-male-release method have been used commonly for the suppression and eradication of newly established populations of *A. ludens* (Tween 1993). Data concerning the age distribution of field populations and the age of released sterile males is necessary to evaluate the overall success of control programs because both feeding and mating behaviors are influenced by age. At the same time the ability to determine the age of released flies would be a useful tool for assessing the quality and longevity of irradiated mass-produced flies.

Research involving age determination of adult flies from the family Tephritidae has been extremely limited. Camin et al. (1991, 1992) applied techniques for age estimation based on accumulation of fluorescent compounds (Mail et al. 1983, Lehane and Mail 1985) in the head of the Mediterranean fruit fly, *Ceratitis capitata* (Wiedemann). The initial results showed age related changes in the intensity of fluorescence obtained from head extracts, but no further studies have been published. Similar studies were performed with the melon fly, *Bactrocera cucurbitae* (Coquillett), by Mochizuki et al. (1993) who demonstrated that the titer of fluorescent compounds in the heads of these insects increased with chronological age.

The great majority of the fluorescent substances in the head capsules of flies are C-6 substituted pterins (Ziegler and Harmsen 1969). These compounds are structurally based upon a pyrazino (2,3-d) pyrimidine ring system and are localized primarily in the eyes. Our previous studies revealed the existence of 10 different pterins in the head capsule of adult *A. ludens* (Tomic-Carruthers et al. 1996). Pilot experiments indicated that overall fluorescence in head extracts changes with age and the titers of two individual pterins (sepiapterin and deoxysepiapterin) increase in correlation with chronological age. Both of these compounds are 6-substituted dihydro pterins. They are fluorescent substances with very similar structure. The only structural difference between these two compounds is found in the side chain at the 6-position. Sepiapterin is 6-lactoyl-7, eight dihydropterin, and deoxysepiapterin is 6-propionyl-7, 8, dihydropterin.

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Their absorption spectra are almost identical and they are both yellow in visible light (Nawa and Forrest 1962).

The main objectives of this work were to test pterins as age specific biological characters compare the rate of accumulation of sepiapterin and deoxysepiapterin under different thermal conditions. Complete high-pressure liquid chromatography (HPLC) separation of these two compounds in individual Mexican fruit fly adults enabled analysis of their age-related accumulation patterns. Because chronological age is not a valid measure of physiological age in insects and other ectothermic animals, all of our experiments were performed at two different constant temperatures in the laboratory and under natural cycling temperatures in the field.

Materials and Methods

Flies were obtained from the United States Department of Agriculture, Agriculture Research Service Subtropical Agricultural Research Laboratory quarantine colonies in Weslaco, TX. They were cultured using established techniques (Rhode and Spishakoff 1965, Spishakoff and Hernandes Davila 1968) under standard laboratory conditions (28°C and a photoperiod of 12:12 [L:D] h). Adult flies were fed a diet containing yeast hydrolysate and sugar and were allowed continuous access to water.

For all laboratory studies and the first field experiment, *A. ludens* adults were kept in cages measuring 20 by 20 by 20 cm. Age of experimental flies was synchronized by placing 250 pupae in cages 3 d before eclosion and removing unemerged pupae 24 h after the first fly emerged. The day of emergence was designated as day 0. Samples for extractions were collected twice a week over the entire experimental period. At the time of sampling, flies were immobilized by cooling at 4°C for ≈1 h, then decapitated and the heads placed individually in vials on silica gel. Vials were immediately wrapped in aluminum foil and stored in a refrigerator at 4°C until chemical analysis was completed.

In the first group of laboratory and field experiments fluorescent compounds were extracted from the head capsule using the procedures of Lehane and Mail (1985). Intensity of fluorescence at 450 nm from the head extracts excited with a 360-nm light was measured on a spectrometer (Perkin Elmer LS50, Shelton CT). The amount of a fluorescent compound is expressed as relative fluorescence based on the direct readings of the spectrometer (Freifelder 1982). Extracts from ≈100 flies were analyzed for each of the tested temperatures (17 and 27°C) and for the field-reared flies. This first field experiment was performed in the previously described small cages, which were placed inside of a large cage built around four grapefruit trees. These cages were located in an orchard in Weslaco, TX. This experiment was conducted during the winter season in the subtropical climate of South Texas. Weather data were recorded hourly within the large field cage using Easy Logger equipment (Omnidata, Shelton, CT).

In the second group of laboratory and field experiments, accumulation of sepiapterin and deoxysepiapterin was measured as a function of chronological time. These pterins were extracted by a modified standard procedure (Tomic-Carruthers et al. 1996) that was originally developed for spectrofluorometric analysis (Lehane and Mail 1985). Extractions were performed in the dark and on ice to prevent oxidation of light sensitive pterins. Pterins were separated by HPLC (Waters, Milford, MA, USA) using 30% methanol as the mobile phase on a reverse phase C18 column (5-μm particle size, 25 cm by 4.6 mm, Alltech Associates, Deerfield, IL, USA). Separated pterins were detected by absorption at 420 nm. Results of the analysis, including quantification of sepiapterin and deoxysepiapterin, were obtained using Millennium 2010 Chromatography Manager Software (Waters). Identification of acquired peaks was accomplished by comparing their UV-absorption spectra using photodiode array (PDA) outputs with spectra of standards. All measurements were evaluated in mVolts as observed using the PDA detector. Sepiapterin and deoxysepiapterin were obtained from Schircks Laboratories, Jona, Switzerland.

Accumulation of sepiapterin and deoxysepiapterin was observed in flies held under two different constant temperature regimes (15 and 30°C) with a photoperiod of 8:16 (L:D) h in environmental chambers. The samples (four male and four female flies) were collected two times a week and processed to determine titers of sepiapterin and deoxysepiapterin as previously described. The sexual maturation of females was followed as an additional measure of adult development. The time of the first oviposition was observed and used as a sign of sexual maturity.

Field experiments were executed during the winter season when *A. ludens* is normally found to be a pest in South Texas. Approximately 5,000 sterile flies were released in a large cage 5,620 m^3 (32 by 48 by 12 feet) enclosing four grapefruit (*Citrus x paradisi*) trees. To ensure survival of the released flies, sugar water was provided in addition to food sources that were naturally available in the cage. Samples were collected approximately once a week depending on the weather. The collections were made by hand in the afternoon while flies were resting on leaves. The flies were then taken to the laboratory, where samples were processed by the procedure described earlier.

Quarantine regulations in South Texas require that sterilized flies be used when experiments are preformed in a single screened field cage. Flies were sterilized by exposing pupae to 7 KRad of radiation from a ^{137}Cs isotope source. Because this additional factor (irradiation) was introduced in this field experiment, a supplementary test was conducted to determine if irradiation had an effect on synthesis and accumulation of deoxysepiapterin and sepiapterin. Irradiated and non-irradiated flies were placed separately in two small cages within the large field cage. They were provided with standard diet and an excess

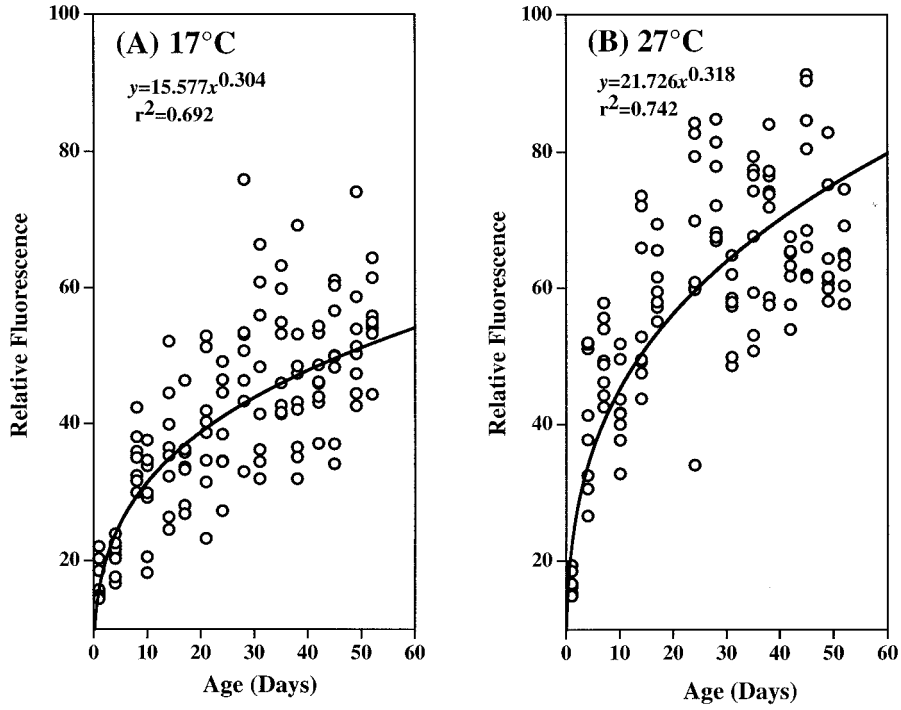

Fig. 1. Relative fluorescence (y) obtained from the head extracts of adult *Anastrepha ludens*, maintained at (A) 17°C [n = 127] and (B) 27°C [n = 120] as a function of chronological age in days (x). Each circle on the graph represents the value obtained from an individual fly.

of water. Samples were collected once a week and processed as described previously.

All statistical analyses were conducted using SYSTAT 5.2 for the Apple Macintosh Computer (Wilkinson 1989). The multivariate general linear hypothesis (MGLH) procedure and its associated significance tests were used to evaluate all data. Regression models were selected based on the form of the observed data and the resulting parameters compared using the MGLH/test feature. Model significance, parameter estimates, and their measures of variation have been provided in the text and associated figures.

Results and Discussion

Fluorescence. In the laboratory, increase of relative fluorescence in the head extracts was variable but positively correlated with the age of the flies (Fig. 1). The best fit was obtained with the model $y = ax^b$ (y = relative fluorescence and x = d postemergence) for each of the analyzed samples as it best describes the underlying biochemical process involved in this chemical accumulation. However, the slope of regression changed with the increase of rearing temperature causing a marked effect on the accumulation of fluorescent substances in the head capsule (Fig. 1). A statistically significant difference ($F = 40.522$; df = 1, 243; $P < 0.001$) was found in the titer of relative fluorescence between individuals reared at the suboptimal (17°C) and optimal (27°C) temperatures (Baker et al. 1944). Although the values of the slopes (0.304 ± 0.0018 and 0.318 ± 0.002) and intercepts were small (15.577 ± 0.87 and 21.726 ± 0.72), both parameters were found to be significant in the overall model due to the small standard errors. In the model $y = ax^b$, slight differences in the exponent parameter (b) make large differences in the fitted regression line (Fig. 1).

The results from the first field experiment were highly variable and accordingly showed a low coefficient of determination between relative fluorescence and the age of the individual fly (Fig. 2A). We note, however, that no samples were collected for adult flies from 1 to 10 d old because weather conditions did not permit collection of material during the first 10 d of the experiment. The average temperature in the field was 16.9°C (with 4.98°C minimal and 27.6°C maximal temperature) during the 40 d of experiment (Fig. 2B); nevertheless, the results showed an insignificant increase ($F = 0.1653$; df = 1, 92; $P = 0.671$) in the relative fluorescence with the chronological age of the flies (Fig. 2A). Our attempt to increase correlation between intensity of relative fluorescence and age by transforming time into degree-days (DD) based on accumulation of field temperatures over a 10°C base temperature (Leyva-Vazquez 1988) did not increase the predictive value of our model in an appreciable way (data not shown).

From 10 to 40 d, linear regression ($y = 0.201x+39.643$, y = relative fluorescence and x = d postemergence) best described the obtained results.

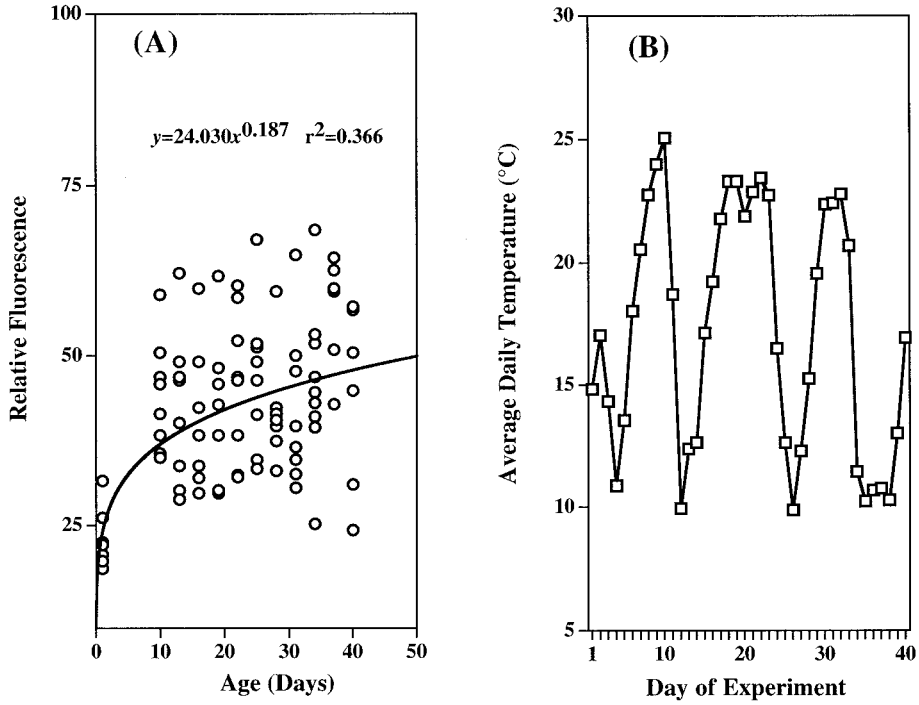

Fig. 2. (A) Relative fluorescence (y), of head extracts from adult adult *Anastrepha ludens* (maintained in small experimental cages in the field) as a function of chronological age in days (x). Each circle on the graph represents the value obtained from an individual fly [$n = 43$]. (B) Temperature conditions recorded in the field during the experiment.

In this case, we found no significant slope ($F = 0.103$; df = 1, 78; $P = 0.742$) and a low coefficient of determination ($r^2 = 0.031$) due to high variability.

HPLC Quantification of Sepiapterin and Deoxysepiapterin. We reported previously that sepiapterin and deoxysepiapterin accumulate in the head capsule of *A. ludens* in correlation with time (Tomic-Carruthers et al. 1996). Experiments with a similar basic design to the fluorescence studies were carried out to determine whether either of these two compounds was suitable for age estimation of *A. ludens.* At the same time, this study compared the dynamics of accumulation of these two compounds in a large number of individual flies held under the same environmental conditions.

In Figs. 3 and 4, each point representing the titer of sepiapterin has a corresponding point representing the titer of deoxysepiapterin from the same individual fly. This allows the direct comparison of these two compounds in relation to their associated accumulation with the age of individual flies. Fig. 3 shows trends in the accumulation of these two compounds in flies reared at 15 and 30°C, respectively. Deoxysepiapterin accumulates with age, and the dynamics of that accumulation depends on temperature. However, values obtained for sepiapterin exhibited poor correlation between age and accumulation of this compound. The nonlinear regression model of $y = ax^b$ (y = relative titer of the test compound in mVolts and x = d postemergence) best describes the accumulation of deoxysepiapterin and sepiapterin, with the equations for the best fit and the coefficients of determination presented in the Fig. 3. At both temperatures (15 and 30°C), sepiapterin showed no significant slope ($F = 0.102$; df = 1, 93; $P > 0.75$ and $F = 0.00661$; df = 1, 45; $P > 0.90$, respectively). Deoxysepiapterin, however, increased significantly ($F = 239.4$; df = 1, 93; $P < 0.0001$ and $F = 56.96$; df = 1, 45; $P < 0.001$) with time at both 15 and 30°C (Fig. 3). Both the slope (0.543 ± 0.031, and 0.493 ± 0.026) intercept (737.9 ± 085.1 and 2,556.5 ± 232.2) parameters were found to be significantly different in the full model.

The life span of *A. ludens* adults was significantly longer in the flies reared at 15°C than those held at 30°C. The first oviposition event in the group reared at 15°C was recorded when flies were 27 d old, while flies reared at 30°C laid their first eggs 7 d after adult emergence. As expected, maturation and aging of the flies were much faster under high temperature conditions.

Results of the field studies on sepiapterin and deoxysepiapterin accumulations are shown in Fig. 4. Titer of deoxysepiapterin increased significantly ($F = 67.64$; df = 1, 36; $P < 0.00001$; $r^2 = 0.632$), whereas sepiapterin did not increase with age ($F = 3.047$; df = 1, 36; $P = 0.10$; $r^2 = 0.078$). Nearly a 10-fold increase in deoxysepiapterin levels was noted over the 28-d experimental period, whereas no increase was seen in sepiapterin levels. The experiment lasted 28 d with an

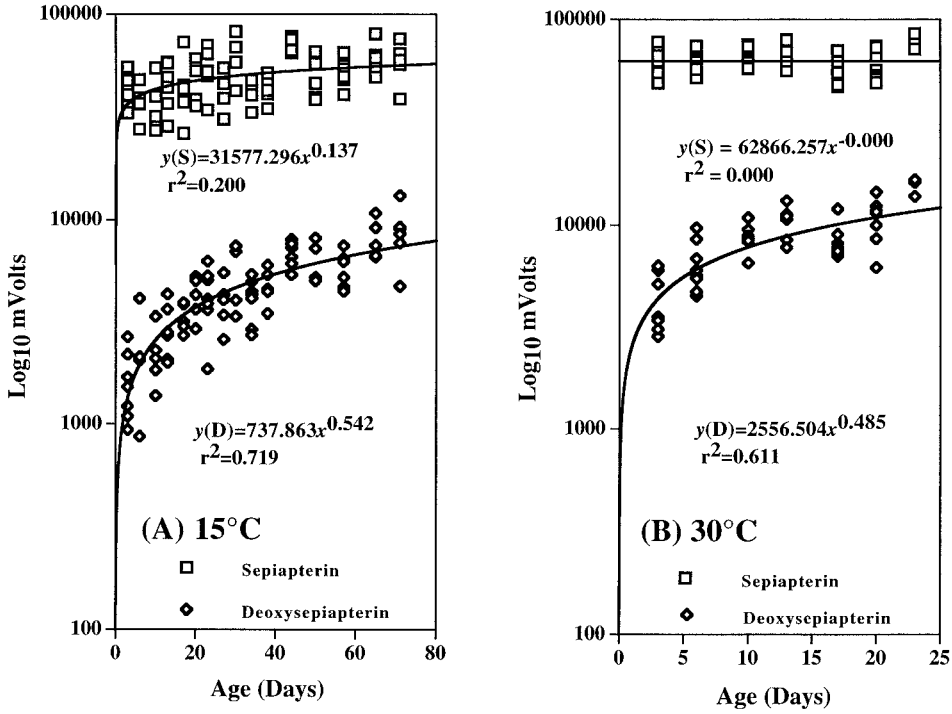

Fig. 3. Accumulation of sepiapterin (squares) and deoxysepiapterin (diamonds) expressed as mVolts (y) as a function of chronological age in d (x) in the heads of adult *Anastrepha ludens* maintained at (A) 15°C [n = 93] and (B) 30°C [n = 48]. Each symbol represents the value obtained from an individual fly.

average temperature of 17°C with minimal and maximal temperatures of 3.1 and 29.7°C, respectively.

In the experiments comparing accumulation of sepiapterin and deoxysepiapterin in irradiated and nonirradiated flies, no significant differences were found in the treatments over the 30-d experimental period (data not shown).

Overall, our laboratory experiments show that the relative fluorescence from the head extracts of *A. ludens* increases with time and that this increase is affected by ambient temperature (Fig. 1). This phenomenon is described and used as an age grading tool in other species of Diptera (Lehane and Mail 1985; Langley et al. 1988; Thomas and Chen 1989; Cheke et al. 1990; Wall et al. 1990, 1991; Camin et al. 1991; Krafsur et al. 1992; Mochizuki et al. 1993). The correlation between temperature, chronological age and the increase of relative fluorescence in *A. ludens* suggests that this occurrence may be biologically linked with physiological aging in this species as well.

Because our laboratory results were generated under constant temperatures in an unnatural environment, field experiments were conducted to test the effect of naturally varying temperature conditions on increase of fluoresce of head extracts in relationship with age. Our field experiment revealed poor correlation between age of the fly and the titer of combined fluorescent compounds taken from the head capsule of the test flies. The pattern of pterin accumulation was not uniform over the entire life span of the flies. The amount of pterins doubled in the first 10 d and then changed little for the rest of the adult life span. These results could not be explained easily by ambient temperature conditions recorded in the field but are similar to accumulation patterns of pterins observed in Anopheles mosquitoes (Wu and Lehane 1999). Average temperature in the first 10 d was 18.2°C followed by average temperature of 16.9°C for the next 27 d. When translated into cumulative degree-days, the calculated values were 81.03 DD for day 10 and 281.1 DD for day 37 when the last samples were collected. Thus, temperature conditions cannot be the sole explanation for the uneven rate of pterine accumulation in this experiment. Also, the variability between flies in relative fluorescence through time made this technique unusable as an age-specific character in *A. ludens.*

The relative intensity of fluorescence in our experiments measures all the fluorescent compounds from the head capsule, not just the specific compounds that may accumulate with age. In our previous studies (Tomic-Carruthers et al. 1996), we were able to separate 10 different pterins in the head capsule of *A. ludens* adults. Our studies revealed that sepiapterin and deoxysepiapterin have different patterns of accumulation. The dynamics of accumulation of sepiapterin in *A. ludens* (Fig. 3) correspond to the pattern described for *Drosophila melanogaster* (Meigen) (Fan et al. 1976). The synthesis of sepiapterin in head capsules of *D. melanogaster* starts before emergence and accumulates for a short period after emergence, usu-

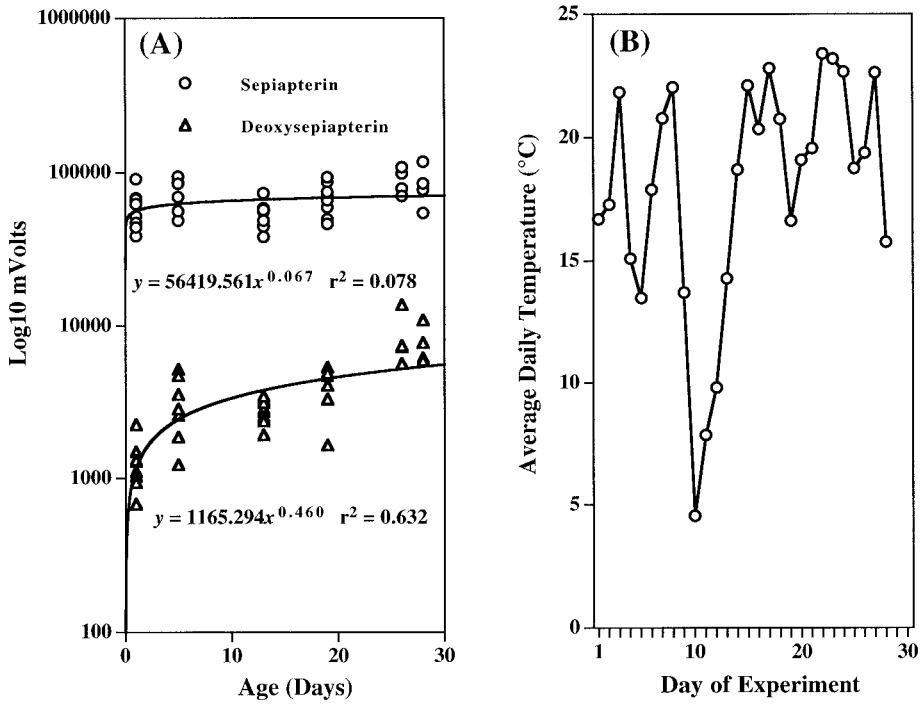

Fig. 4. (A) Accumulation of sepiapterin (squares) and deoxysepiapterin (diamonds) expressed as mVolts (y) as a function of chronological age in d (x) in the heads of adult *Anastrepha ludens* reared on grapefruit trees in a field cage. Each symbol represents the value obtained from an individual fly [n = 39]. (B) Temperature conditions recorded in the field during experiment.

ally 2–4 d, depending upon temperature. At this point, sepiapterin levels reach a maximum and remains relatively constant for the rest of the life span. Data on the accumulation of deoxysepiapterin are not available in *D. melanogaster* or any species except for *A. ludens* (this study).

Synthesis of deoxysepiapterin in the heads of *A. ludens* begins at the time of adult emergence (Tomic-Carruthers 1997). Accumulation continues over the entire life span. This accumulation is positively correlated with ambient thermal conditions, and it reflects physiological aging. We know of no other pterin with a similar pattern of accumulation in the head capsule of *A. ludens.*

Age-specific biological characters are defined "as those [factors] which reflect changes in physiological functions or composition of an organism rendering death a more likely occurrence" (Donato et al. 1979). In a practical sense, the determination of age-specific characters is important because they can be used as a tool for physiological age estimation of animals in natural environments. Our experiments proved that the "standard method" that uses relative fluorescence from the head capsule of flies as a parameter for age estimation is not adequate for *A. ludens.* We have tested two individual pterins as potential candidates for age specific biological parameters and found deoxysepiapterin is an appropriate biological character for age estimation. Our results show an increase in deoxysepiapterin levels in the head capsule of flies with age, and predictive equations (Figs. 2A and 3) obtained in our experiments (r^2 values of 0.719 for 15; 0.611 for 30°C and 0.660 for field conditions) are significant. Unexplained variation is present in this as in all previous studies involving the use of relative fluorescence as age parameter.

Explanations for individual variations obtained in the populations under study are critical in experiments aimed at identifying age-specific biological characters Only by understanding the cause of this variation can further improvements in prediction be made. Based on the analytical method proposed by Donato et al. (1979), part of the variation in our experiments could be the result of selective mortality that naturally occurs in populations. Therefore, as the population ages, samples taken from experimental populations progressively represent survivors that age more slowly than the rest of the population. This explanation fits well with the aging profile obtained in our studies, because all our experiments showed increasing variability with age. Unfortunately, we did not collect mortality data in our experiments, nor do we have data on the average life span of Mexican fruit flies under our experimental conditions. Additional research on the dynamics of fruit fly aging and population mortality assessments are required. Nevertheless, this is the first study demonstrating that the accumulation pattern of deoxysepiapterin depends on the physiological age of the Mexican fruit fly and that

this compound could be used as an age specific biological parameter in the field.

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